Fold

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/85, 5/10, C12Q 1/68, A61K 35/00 — A1

(11) International Publication Number: **WO 00/39314**

(43) International Publication Date: 6 July 2000 (06.07.00)

(21) International Application Number: PCT/CA99/01235

(22) International Filing Date: 23 December 1999 (23.12.99)

(30) Priority Data:
9828709.7 24 December 1998 (24.12.98) GB

(71) Applicant *(for US only)*: NOVATION PHARMACEUTICALS INC. [CA/CA]; 1323 Regan Avenue, Coquitlam, British Columbia V3J 3B6 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KASTELIC, Tania [CA/CA]; 1323 Regan Avenue, Coquitlam, British columbia V3J 3B6 (CA). CHENEVAL, Dominique [CH/CA]; 1323 Regan Avenue, Coquitlam, British Columbia V3J 3B6 (CA).

(74) Agents: ROBINSON, Christopher, J. et al.; Smart & Biggar, Vancouver Centre, Suite 2200, 650 West Georgia Street, P.O. Box 11560, Vancouver, British Columbia V6B 4N8 (CA).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU. LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ASSAY FOR IDENTIFYING COMPOUNDS WHICH AFFECT STABILITY OF mRNA

(57) Abstract

A method is provided for the identification of a compound which affects mRNA stability, in particular induces mRNA degradation, in which a DNA expression system which in the absence of test compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control. The method may be used to identify compounds which induce degradation of mRNA, e.g. cytokine (e.g. IL-1β) mRNA, which when inappropriately stabilised can give rise to diseases or medical conditions, e.g. cytokine induced inflammatory disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

ASSAY FOR IDENTIFYING COMPOUNDS WHICH AFFECT STABILITY OF mRNA

This invention relates to an assay for the identification of biologically active compounds, in particular to a reporter gene assay for the identification of compounds which have an effect on mRNA stability.

Recently, it has become increasingly apparent that the regulation of RNA half-life plays a critical role in the tight control of gene expression and that mRNA degradation is a highly controlled process. RNA instability allows for rapid up- or down-regulation of mRNA transcript levels upon changes in transcription rates. A number of critical cellular factors, e.g. transcription factors such as c-myc, or gene products which are involved in the host immune response such as cytokines, are required to be present only transiently to perform their normal functions. Transient stabilisation of the mRNAs which code for these factors permits accumulation and translation of these messages to express the desired cellular factors when required; whereas, under non-stabilised, normal conditions the rapid turnover rates of these mRNAs effectively limit and "switch off" expression of the cellular factors. However, abnormal regulation of mRNA stabilisation can lead to unwanted build up of cellular factors leading to undesirable cell transformation, e.g. tumour formation, or inappropriate and tissue damaging inflammatory responses.

Although the mechanisms which control mRNA stability are far from understood, sequence regions have been identified in a number of mRNAs, which appear to confer instability on the mRNAs which contain them. These sequence regions are referred to herein as "mRNA instability sequences". For example, typical mRNA instability sequences are the AREs (AU rich elements), which are found in the 3'UTR (3' untranslated region) of certain genes including a number of immediate early genes and genes coding for inflammatory cytokines, e.g. IL-1β and TNFα.

As described in our copending British patent applications no. 9828707.1 and 9828710.5, we have discovered compounds which promote instability of mRNAs which contain mRNA instability sequences. Such compounds may be used to induce degradation of mRNAs, thus preventing or reversing inappropriate mRNA accumulation and thereby decreasing or preventing unwanted protein, e.g. cytokine, expression. Thus such compounds are potentially useful

pharmaceutically for prophylaxis or treatment of diseases or medical conditions which involve inappropriate mRNA stabilisation and accumulation and resultant undesirable protein expression.

The present invention relates to a reporter gene assay for identifying compounds which affect the stability of mRNAs which contain mRNA instability sequences.

Accordingly the present invention provides a method for the identification of a compound which affects mRNA stability, in which a DNA expression system which in the absence of the test compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control.

Preferably the method of the invention is adapted for the identification of compounds which promote instability of mRNAs which contain mRNA instability sequences. The reporter gene assay may be used to screen individual compounds and libraries of compounds, including combinatorial compound libraries. The reporter gene assay may be used as a first line screening assay to identify lead compounds and may be used to compare or quantify the mRNA instability promoting activity of compounds, e.g. to compare compounds produced from medicinal chemistry lead optimisation/ derivatisation programmes.

Thus in preferred embodiments the invention provides

i) a method for the identification of a compound which induces mRNA degradation, comprising contacting the compound with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control, or

ii) a method for the comparison of compounds which induce mRNA degradation, comprising separately contacting the compounds with a DNA expression system which in the absence of the compounds is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

The DNA expression system typically comprises a gene coding for expression of the protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements including promoter and/or enhancer regions, and characteristically DNA corresponding to at least one copy of a mRNA instability sequence. Appropriate choice of promoter/enhancer sequences and other expression control sequences is a matter well within the ambit of the skilled worker in the art, and does not form a substantive part of the invention. Thus, for instance, for expression in mammalian cells a viral promoter such as an SV40, CMV or HSV-1 promoter may be used. On the other hand appropriate choice of mRNA instability sequence is of importance to the successful functioning of the reporter gene assay and forms part of the invention.

Thus in a further aspect the invention provides a reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

mRNA instability sequences have been identified in the UTRs, in particular the 3' UTRs, of a large number of transiently expressed genes including genes for cytokines, chemokines, nuclear transcription factors, protooncogenes, immediate early genes, cell cycle controlling genes, oxygenases, genes involved in and controlling of apoptosis. The natural RNA sequences which comprise the mRNA instability sequences are alternatively referred to as adenylate/uridylate (AU)-rich elements, or AREs. Transiently expressed genes which contain mRNA instability sequences include, for example, the genes coding for GM-CSF, c-*fos*, c-*myc*, c-*jun*, *krox*-20, *nur*-77, *zif268*, bcl-2, β-IFN, uPA, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, TNF-α, MCP1, syn1, β_2-AR, E-selectin, VCAM-1, ICAM-1, Gro-α, Gro-β, MMP-1, MMP-2, collagenases, P-glycoproteins (MDR), MRPs, Pγh1 (pf mdr), COXII, and MIP-2α.

The following publications include extensive discussion of mRNA instability sequences and AREs, the sequences motifs which they contain and (minimum) sequence requirements for

mRNA destabilisation, as well as identifying a number of mRNA instability sequences and the genes which contain them:

Shaw & Kamen, Cell, Vol. 46, 659-667, August 29 1986 (GM-CSF);

Shyu et al., Genes & Development, 5:221-231 (1991) (c-*fos*);

Sachs, Cell, Vol. 74, 413-421, August 13 1993 (Review. "Messenger RNA Degradation in Eukaryotes");

Chen et al., Mol. Cell. Biol., Jan 1994, p 416-426 (c-*fos*);

Akashi et al., Blood, Vol. 83, No. 11, (June 1), 1994: pp 3182-3187 (GM-CSF etc.);

Nanbu et al., Mol. Cell. Biol., July 1994, p. 4920-4920 (Upa);

Stoecklin et al., J. Biol. Chem., Vol. 269, No. 46, November 18 1994, pp 28591-28597 (IL-3);

Lagnado et al., Mol. Cell. Biol., Dec. 1994, p. 7984-7995 (general);

Zhang et al., Mol. Cell. Biol., Apr. 1995, p. 2231-2244 (yeast);

Zubiaga et al., Mol. Cell. Biol., Apr. 1995, p. 2219-2230 (general);

Winstall et al., Mol. Cell. Biol., July 1995, p. 3796-3804 (c-*fos*, GM-CSF);

Chen et al., Mol. Cell. Biol., Oct. 1995, p. 5777-5788 (c-*fos*, GM-CSF);

Chen et al., TIBS 20 November 1995, 465-470 (review);

Levy et al., J. Biol. Chem., Vol. 271, No. %, February 2 1996, pp. 2746-2753 (VEGF);

Kastelic et al., Cytokine, Vol. 8, No. 10 (October), 1996: pp751-761;

Crawford et al., J. Biol. Chem., Vol. 272, No. 34, August 22 1997, pp. 21120-21127 (TNF-α);

Xu et al., Mol. Cell. Biol., Aug. 1997, Vol. 18, No. 8, p. 4611-4621 (general);

Danner et al., J. Biol. Chem., Vol.273, No. 6, February 6 1998, pp. 3223-3229 (human β_2-Adrenergic Receptor);

Lewis et al., J. Biol. Chem., Vol. 273, No. 22, May 29 1998, pp. 13781-13786 (TNF-α);

Chen, C.-Y. and Shyu, A.-B. Mol. Cell. Biol. Vol.14, No.12, 1994, pp. 8471-8482; and

Klausner, R. et al., Cell, Vol. 72, 1993, pp. 19-28.

As described in the above publications mRNA instability sequences often contain one or more copies of sequence motifs, e.g. selected from:

AUUUA; UAUUUAU; UUAUUUA(U/A)(U/A), and AUUUAUUUA.

Thus mRNA instability sequence for use in the invention usually contains at least 1, preferably at least 2, or more preferably at least 3 of such sequence motifs or parts thereof (e.g. normally

containing at least 4 consecutive nucleotides from the motif) in appropriate juxtaposition, normally together, e.g. as tandem repeats, or with other, e.g. intervening, RNA sequences. Typically the mRNA instability sequence comprises from about 20 up to about 100 or more, preferably from about 30 to about 50, nucleotides in length. The mRNA instability sequence may be derived as a restriction fragment from the 3' UTR of an appropriate gene, or as a de novo synthesised nucleotide sequence. Alternatively the whole or a substantial part of the 3' UTR of an appropriate natural gene sequence, which contains a mRNA instability sequence may be used.

DNA corresponding to any mRNA instability sequences or AREs, including those described in the above publications, or functionally equivalent variants thereof, may be used in the DNA expression system of the invention. Preferably, however, the mRNA instability sequence used is one derived from the mRNA which codes for a protein which is implicated in the disease of interest. Thus, for example, a mRNA instability sequence for use in detecting compounds which destabilise the mRNA which codes for a cytokine or oncogene which is involved in the aetiology of a particular disease process, is preferably derived from the gene which codes for the cytokine or oncogene in question, e.g. lead compounds for treatment of IL-1 mediated diseases, such as rheumatoid arthritis or osteoarthritis are preferably detected using a reporter gene expression system comprising an IL-1 mRNA instability sequence.

Thus by way of illustration of the invention a preferred mRNA instability sequence for use in the identification of compounds which destabilise IL-1β mRNA is derived from the 3' UTR of IL-1β mRNA, e.g. the sequence shown in Figure 1. More preferably the IL-1β mRNA instability sequence may comprise a fragment of the 3' UTR of IL-1β mRNA. For example, a particularly preferred IL-1β mRNA instability sequence comprises the 30 nucleotide sequence derived from the 3' UTR of IL-1β mRNA (shown in Figure 2).

Preferably the mRNA instability sequence is located in the 3' UTR of the reporter gene. Thus for example, the DNA sequence corresponding to the mRNA instability sequence is inserted as or as part of an appropriate DNA segment into a suitable restriction site in the 3' UTR of the native reporter gene.

The DNA expression system is preferably a cell based expression system, conveniently in the form of a suitably transformed cell line, preferably a stably transformed cell line. The host

cell is typically an eucaryotic host cell, in particular an animal host cell, especially a mammalian host cell.

Preferably the host cell is of the same general cell type as the cells which express the protein which is coded for by the mRNA which it is desired to destabilise. Thus for instance, if the assay of the invention is to be used for the identification of compounds which destabilise the mRNA coding for a cytokine, the host cell used is preferably a cell or cell line which is of the same or similar cell type to the cells which normally produce the cytokine in question. For example, monocyte or monocyte-like cell lines may be used as host cells for assaying for compounds which destabilise cytokine, e.g. IL-1β, mRNA. Preferred cell lines for oncogene and other cancer related gene mRNA instability assays are, e.g. Colon 205, KB 31, KB 8511, DU-145, HCT116, MCF7, MCF7/ADR, MDA-MB-231, MDA-MB-435 and MDA-MB-435/TO. Particularly preferred cell lines for use as the host cells in assays of the invention for identification of compounds which destabilise cytokine, e.g. IL-1β, mRNA are the THP-1 cell line (for instance as described by Auwerx J. (1991), Experientia, 47: 22-30) and similar monocytic, e.g. human leukaemia, cell lines.

Preferably also, the mRNA instability sequence and the host cell are derived from the native mRNA which it is desired to destabilise and the native cell type in which that mRNA is produced respectively. Thus for instance, for identification of compounds which destabilise cytokine mRNA, the mRNA instability sequence is preferably derived from the mRNA which codes for the cytokine in question and the host cell is preferably of the same cell type as the native cell type in which the cytokine mRNA is produced. For example, for identification of compounds which destabilise IL-1β mRNA, the mRNA instability sequence is preferably derived from the 3' UTR of IL-1β mRNA and the host cells used are monocyte-type cells, e.g. THP-1 cells.

Although the mechanism of mRNA destabilisation, and the role of mRNA instability sequences in this, is not fully understood, it is clear that the presence of other factors besides the destabilising compound and the mRNA instability sequence are required for mRNA destabilisation to take place; for instance, as discussed in previously identified literature references. Conveniently such other factors are provided by the transformed host cell environment and complement or complete the interaction of the compound and the mRNA

instability sequence to effect destabilisation of the mRNA. Preferably the transformed host cells may be stimulated or otherwise activated to enhance mRNA destabilisation, e.g. to provided enhanced levels of the cellular factors required for mRNA destabilisation. In particular we have found that improved results are obtained in the assay of the invention if differentiated transformed host cells are used. For example, in the case of transformed THP-1 cells we have found that the best results are obtained if the transformed THP-1 cells are grown, differentiated and stimulated with γIFN and LPS as is normal for THP-1 cells, e.g. as described hereinafter in the Examples.

The protein coded by the reporter gene mRNA may itself comprise the detectable signal. For instance, the protein may comprise a fluorescent protein, e.g. green fluorescent protein. Preferably, however, the protein is such that it is capable of reacting with an appropriate substrate or other substance to give a detectable signal. Conveniently the protein coded by the mRNA is an enzyme or enzymically active fragment of an enzyme. Examples of suitable enzymes include horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT), alkaline phosphatase (AP), secreted alkaline phosphatase (SEAP), β-galactosidase, or especially luciferase. Methods for detecting and determining such enzymes are well-known, using appropriate substrates and measurements; for instance, as described hereinafter for the determining the levels of luciferase expression. It will be appreciated, however, that any suitable detectable protein and measurement procedure may be used.

In the assay of the invention the presence of a compound which destabilises mRNA is indicated by a decrease in the magnitude of the detectable signal given by the protein produced from the expression system in the presence of the compound as compared with a control; destabilisation of the reporter gene mRNA by the compound leads to a decrease in expression of the protein and thus a decrease in the magnitude of the signal. A suitable control for use in the assay of the invention comprises a DNA expression system which corresponds to the reporter gene DNA expression system, i.e. contains sequence coding for expression of the detectable protein but which does not contain sequence corresponding to a mRNA instability sequence. Preferably the control DNA expression system is identical to the reporter gene expression system except that the DNA corresponding to the mRNA instability sequence has been removed, deleted or otherwise disabled as a mRNA instability sequence. Preferably the control DNA expression

system is also in the form of a transformed cell line, typically a stably transformed cell line derived from the same host cell line, e.g. a THP-1 cell line, as the reporter gene transformed cell line.

Accordingly in a preferred embodiment the invention provides an assay system for the identification of compounds which destabilise mRNA comprising

a reporter gene DNA expression system as defined above, and

a control DNA expression system which comprises a gene coding for expression of the protein having the detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking a functional mRNA instability sequence.

Preferably both the reporter gene DNA expression system and the control DNA expression system are in the form of stably transfected cell lines.

Alternatively the reporter gene expression system may be tested in the presence and absence of the test compound, testing in the absence of the test compound being used as the control. In another alternative embodiment a control DNA expression system may also be present in the same cell line as the reporter gene DNA expression system. The control DNA expression system in this case would code for a detectable protein which is different than the protein coded for by the reporter gene expression system, and as before, the control DNA expression system lacks any functional mRNA instability sequence.

The invention is further described by way of illustration of the invention only in the following Examples which relate to a particular assay of the invention and refer to the accompanying Figures:

Figure 1 which shows the DNA sequence of IL-1β 3' UTR;

Figure 2 which shows the 30 bp fragment used as a mRNA instability sequence in Example 1;

Figure 3 which shows plasmid diagrams for pGL2_Neo30 and pGL2-Control;

Figure 4 which shows graphs of luciferase activity over the time of differentiation for clone No. 53 (A) and clone No. 63 (B);

Figure 5 shows graphs of luciferase half lives, 4 and 8 hours after addition of compounds for clones 53 and 63 treated with radicicol analog A (SDZ 216-732), actinomycin D (act D.) and cyclohexamide (CHX);

Figure 6 shows graphs of luciferase activity from clones 53 (solid bars) and 63 (open bars) treated with various concentrations of radicicol analog A (SDZ 216-732);

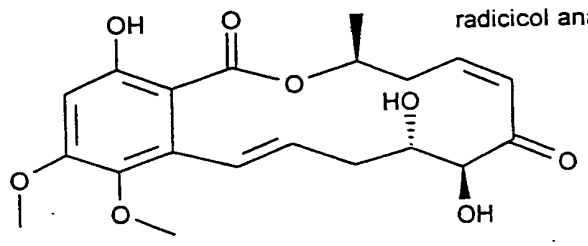
Figure 7 shows graphs of luciferase activity for undifferentiated (undiff) and differentiated (diff) clone 53 (solid bars) and clone 63(open bars) treated with radicicol analog A, and

Figure 8 shows a graph of the concentration inhibition of luciferase activity by radicicol analog A.

EXAMPLES

We have shown earlier (Kastelic et al., CYTOKINE. Vol. 8, No. 10 (October), 1996: pp751-761) that radicicol analog A (the compound shown below) confers mRNA instability through the AU-rich element (ARE) motifs located in the 3' untranslated region (3' UTR) of genes subject to mRNA instability. For these studies, the segment of 3' UTR of IL-1β which contains all the AREs was deleted and the resulting IL-1β -AU cDNA was subcloned into an expression vector. Stably transfected THP-1 cells containing this construct were analyzed by the RNase protection method (Kastelic et al. ibid) and showed resistance of the AU-less derived RNA towards radicicol analog A.

The 3'UTR of IL-1β mRNA contains a total of 6 AUUUA motifs three of which are in tandem (see Figure 1). For the construction of the luciferase reporter gene assay, we used only the a fragment comprising of the underlined sequence shown in Fig. 1 which contains three tandem repeats. Findings by Zubiaga et al (ibid) indicate that the minimal sequence of the mRNA instability motif is UUAUUUAUU (a sequence which occurs in the inserted 30 bp IL-1β fragment which we used) rather than just AUUUA alone.



radicicol analogue A

Example 1: Construction of pGL2_Neo30 and stable cell lines

In order to obtain a vector for stable integration into THP-1 cells, a XhoI - SalI fragment of the neo resistant gene (expressing aminoglycoside 3' phosphotransferase) derived from pMCIneo (Stratagene) is subcloned into the SalI site of pGL2-Control (Promega). This resulting plasmid was called pGL2_Neo. A 30bp fragment (containing three tandem AUUUA motifs, based on the IL-Iβ 3'UTR sequence) obtained by annealing two complementary synthetic oligonucleotides (see Figure 2) is subcloned into pGL2_Neo using the PflM1 restriction site. This results in the luciferase expression vector pGL2_Neo30 (Fig. 3). Fig. 2 shows the IL-1β 3'UTR sequence containing three tandem AUUUA motifs used for ligation into the PflMI site of pGL2_Neo. Expression vector pGL2-β-galactosidase (Promega) has the *lacZ* gene driven by the same promoter (SV40) as the luciferase gene in pGL2_Neo30 and pGL2_Neo, but plasmid pGL2-β-galactosidase does not contain any mRNA instability sequences.

THP-1 cells are then transfected with pGL2_Neo vector (to generate control cell lines) or are cotransfected with pGL2_Neo30 vector pGL2-β-galactosidase by electroporation. 10^7 cells/ml in 1.3mM KH_2P0_4, 7.36m.M Na_2HP0_4, 2.44mM KCl, 124mm NaCl, 5mM glucose, 9.6μM $MgCl_2$ and 16μM $CaCl_2$ pH 7.2 are transfected with 20μg of DNA in a Bio-Rad Gene Pulser (250V, 690μF and indefinite resistance) using a 0.4cm cuvette. Cells are subsequently cultured in RPMI medium containing 10%FBS, 2mM L-Gln (L-glutamine), 50μM 2-mercaptoethanol and 600μg/ml of G418 (geneticin). After transfection of pGL2_Neo30 and pGL2_Neo into THP-1 cells, stable cell lines are obtained by selection for G418 resistance and assayed for luciferase activity (and the cotransfected cell line is also assayed for β-galactosidase activity which can serve as an internal control – see Example 5 below). One cell line of each

transfection is chosen for further analysis; the pGL2_Neo30/ pGL2-β-galactosidase cell line is referred to as clone No. 63 and the pGL2_Neo cell line as clone No. 53. No endogenous luciferase activity could be detected in normal THP-1 cells.

The tissue culture and luciferase activity measurements are carried out as described below.

Tissue culture:

The transfected human monocytic leukaemia cell lines, clones No. 53 and 63 are grown in RPMI medium supplemented with 110 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Gln and 2 g/1 $NaHCO_3$. Heat-treated FBS (5%) is added before use. The cells are grown to a density of 5x 10^5/ml and induced to differentiate with 100 U/ml (final concentration) γIFN. Three hours later, 10 µl of LPS (5µg/ml final concentration) is added. This time point is designated time 0. Compounds are added at various times after LPS addition as indicated.

Luciferase activity measurement:

In order to adapt the system to the use of 96 well plates, cells are grown in Packard flat bottom white polystyrene microplates (Cat. No.6005180) in RPM1 medium lacking phenol red (AMIMED). Cells are plated at 5x10^4/well. After treatment of the cells, luciferase is measured using the Packard Luc Lite system (Cat. No.601691 1) according to the manufacturer's instructions in a final volume of 205µl. Briefly, to a cell suspension of 5 x 10^5 cells/ml, γIFN (1000U/ml Boehringer Mannheim No. 1050494) to a final concentration of 100 U/ml and 0.25% (v/v) Luc Lite Enhancer is added. After a 3 hour incubation LPS (50µg/ml SIGMA L-8274) is added to give 5µg/ml final concentration. The cells are then plated at 5x10^4/100µl/well into flat bottom white polystyrene microplates (Packard, Cat. No. 6005180) and incubated for 16 hours. 5 µl of compound solution or control vehicle is then added and the cells are further incubated as indicated. 100 µl of luciferase substrate solution is added and the plates are covered with TopSeal-A press-on adhesive sealing film (Packard Cat.No. 6005185) before measuring

luminescence with a Packard Top Count Scintillation Counter at 22°C. The luciferase signal is stable for at least 90 min.

The differentiation-dependent induction of luciferase activity in the two cell lines, Nos. 53 (A) and 63 (B) are tested and the results obtained are shown in Figs. 4 A and B. In both clones a distinct induction of luciferase expression can be observed, maintaining high levels of activity throughout the time of the assay. This elevated and constant expression of luciferase should be born in mind when analyzing effects of compounds inducing mRNA instability. mRNA degradation will be in constant competition with de novo transcription, unlike the situation in wild-type THP-1 cells were in the case of IL-1β-mRNA, highest levels are obtained 16 hours after LPS addition. One would expect in the case of luciferase to see a weaker effect of mRNA destabilizing drugs since transcription remains high. Indeed this is what we observe in the case of radicicol analog A, see below.

Example 2: Half life of Luciferase mRNA and protein

To measure mRNA degradation using luciferase protein activity it is important to know the half life of the luciferase enzyme in order to determine an optimal time for assaying for potential mRNA destabilizing agents by way of luciferase protein stability. The possibility exists that mRNA could be degraded but due to a long half life of the protein, high enzyme activities could persist. Therefore we analyzed luciferase activities after addition of the transcriptional inhibitor actinomycin D (act. D) or the translational inhibitor cycloheximide (CHX). Fig. 5 shows that in the presence of 20μg/ml act.D as well as in the presence of 20 μM CHX, luciferase activities rapidly decline and after 8 hours of incubation reach a level comparable to the inhibition achieved by radicicol analog A. In view of this relatively short half life of the luciferase enzyme, it is safe to assess any substance for activity on mRNA degradation as early as 8 hours after compound addition.

Example 3: Effect of the radicicol analog A

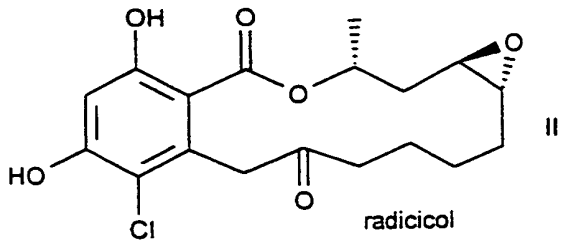
The THP-1 cell lines, clone No. 63 (containing pGL2_Neo30) and clone No. 53 (containing pGL2-Neo) are grown, differentiated with γIFN and stimulated with LPS identical to normal THP-1 cells. Radicicol analog A is added 16 hours after the addition of LPS and cell extracts are then taken 8 hours later or as indicated. Luciferase activity is inhibited by 1 μM radicicol analog A on average by 50% +/-17%, in some cases inhibition was as great as 93%, whereas up to $5x10^{-6}$M of radicicol analog A has no effects on the control clone No. 53, Fig. 6 (solid bars indicate clone No. 53, open bars clone No. 63).

Interestingly, undifferentiated (undiff) clone No. 63 (open bars) when treated with radicicol analog A showed only a limited reduction of luciferase activity (Fig. 7, solid bars indicate clone No. 53), which is either due to the lower expression of luciferase or is indicative of the involvement of a differentially expressed or modified component in the mRNA degradation process mediated by AU-rich elements. Indeed, gel retardation experiments using 241 bp of the AU-rich 3' UTR of IL-1β as a riboprobe showed the binding of additional proteins with γIFN induced differentiation or modification (not shown).

Concentration dependent inhibition of luciferase activity is shown in Fig. 8. Concentrations of radicicol analog A higher than $5x10^{-6}$M also inhibited the control clone due to cytotoxicity or inhibitory activity on transcription.

Example 4: Application of assay to a number of selected substances

A number of selected substances are tested for their activity in the assay of the invention substantially as described in Example 3 (for differentiated cells). The results obtained are given in the Table 1 below. Radicicol (see formula II below) and radicicol analog A show a clear effect on mRNA stability; other compounds tested did not show activity in the assay used.



radicicol

TABLE 1

COMPOUND	Luciferase activity (% of control)	
	clone No. 53	clone No. 63
peptidic ICE inhibitor	87	104
stemphon	95	90
radicicol	98	47
(17α)-23-(E)-dammara-20,23-dien-3β,25-diol	116	91
radicicol analog A	120	49
thalidomide	98	112
dexamethasone	72	63
cyclosporin A	82	74

Example 5: Application of assay using a single cell line

In the previous examples, test compounds are assayed by comparing their activity in two separate cell lines (clone 53 and clone 63). However, clone 63 was cotransfected with two separated plasmids: one plasmid (pGL2_Neo30) contains the luciferase gene with the 30 bp instability sequence driven by the SV40 promoter and the other plasmid (pGL2-β-galactosidase) contains the *lacZ* gene driven by the SV40 promoter but contains no mRNA instability

sequences. The β-galactosidase activity of this cell line should not be effected by exposure of the cells to compounds which promote mRNA instability via mRNA instability sequences. As a result, one should be able (in theory) to screen for compounds having mRNA instability activity by simply comparing luciferase activity in unstimulated cells versus stimulated cells and comparing the β-galactosidase activity in these same cells. To test this hypothesis, the effect of radicicol analog A on luciferase activity and β-galactosidase activity in clone 63 (stimulated and unstimulated cells) was compared to the effect of radicicol analog A on stimulated and unstimulated cells of clone 63 and clone 53. The assay was performed as described in the previous Examples. Table 2 shows the luciferase activities of various concentrations of radicicol analog A in γIFN/LPS stimulated and unstimulated cells of clones 63 and 53. Activities are given in % of control and are based on means of three independent experiments controlled for cell numbers. Table 3 shows the β-galactosidase activities in stimulated and unstimulated cells of clone 63. Activities are given in % of control and are based on means of three independent experiments controlled for cell numbers. It is clear from the data that both the assay of Table 2 and that of Table 3 would have identified radicicol analog A as an active compound.

TABLE 2

Luciferase activity				
	clone 63		clone 53	
	unstimulated	γIFN/LPS stimulated	unstimulated	γIFN/LPS stimulated
	(%control)	(%control)	(%control)	(%control)
No compound	100	100	100	100
1 μM radicicol analog A	63	7	nd	88
10 μM radicicol analog A	11	2	87	63

TABLE 3

β-galactosidase activity				
	clone 63		clone 53	
	unstimulated	γIFN/LPS stimulated	unstimulated	γIFN/LPS stimulated
	(%control)	(%control)	(%control)	(%control)
No compound	100	100	100	100
1 μM radicicol analog A	96	97	99	98
10 μM radicicol analog A	84	70	103	62

CLAIMS

1. A method for the identification of a compound which affects mRNA stability, in which a DNA expression system which in the absence of the test compound is capable of expressing a protein having a detectable signal, and wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control.

2. A method according to claim 1, for the identification of a compound which induces mRNA degradation, comprising contacting the compound with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control.

3. A method for the comparison of compounds which induce mRNA degradation, comprising separately contacting the compounds with a DNA expression system which in the absence of the compounds is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

4. A reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences

comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

5. A stably transfected cell line comprising a reporter gene DNA expression system according to claim 4.

6. An assay system for the identification of compounds which destabilise mRNA comprising

a reporter gene DNA expression system as defined in claim 4, and

a control DNA expression system which comprises a gene coding for expression of the protein having the detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking any functional mRNA instability sequence.

7. An assay system comprising

a stably transfected cell line according to claim 5, and

a stably transfected cell line comprising a control DNA expression system as defined in claim 6.

8. A stably transfected cell line comprising a reporter gene DNA expression system according to claim 4 and a control gene DNA expression system, said control gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal which is different than the protein of the reporter gene DNA expression system and wherein said control gene DNA expression system comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking any functional mRNA instability sequence.

9. An assay system comprising a stably transfected cell line according to claim 8.

10. A compound which destabilises mRNA when identified by a method according to any one of claims 1-3, or by use of a DNA expression system according to claim 4, a cell line according to claim 5 or 8, or an assay system according to claim 6, 7 or 9.

11. Use of a compound according to claim 10 for the prophylaxis or treatment of a disease or medical condition which involves inappropriate mRNA stabilisation and/or accumulation and undesirable protein expression.

* GGACCAAAGG CGGCCAGGAT ATAACTGACT TCACCATGCA

ATTTGTGTCT TCCTAAAGAG AGCTGTACCC AGAGAGTCCT

GTGCTGAATG TGGACTCAAT CCCTAGGGCT GGCAGAAAGG

GAACAGAAAG GTTTTTGAGT ACGGCTATAG CCTGGACTTT

CCTGTTGTCT ACACCAATGC CCAACTGCCT GCCTTAGGGT

AGTGCTAAGA GGATCTCCTG TCCATCAGCC AGGACAGTCA

GCTCTCTCCT TTCAGGGCCA ATCCCAGCCC TTTTGTTGAG

CCAGGCCTCT CTCACCTCTC CTACTCACTT AAAGCCCGCC

TGACAGAAAC CAGGCCACAT TTTGGTTCTA AGAAACCCTC

CTCTGTCATT CGCTCCCACA TTCTGATGAG CAACC GCTTC

CCT ***ATTTATTTATTTA*** TTTG TTTGT TTGTT TTGATTCATT

GGTCTA ***ATTTA*** TTCAAAGGG GGCAAGAAGT AGCAGTGTCT

GTAAAAGAGC CTAGTTTTTA ATAGCTATGG AATCAATTCA

ATTTGGACTG GTGTGCTCTC TTTAAATCAA GTCCTTTAAT

TAAGACTGAA AATATATAAG CTCAGATT ***ATTTA*** AATGGGA

AT ***ATTTA*** TAA ATGAGCAAAT ATCATACTGT TCAATGGTTC

TCA **AATAAA** C TTCACT

FIGURE 1

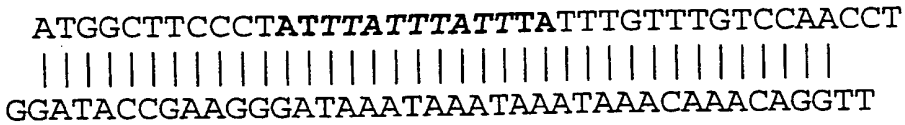

ATGGCTTCCCTATTTATTTATTTATTTGTTTGTCCAACCT
GGATACCGAAGGGATAAATAAATAAATAAACAAACAGGTT

FIGURE 2

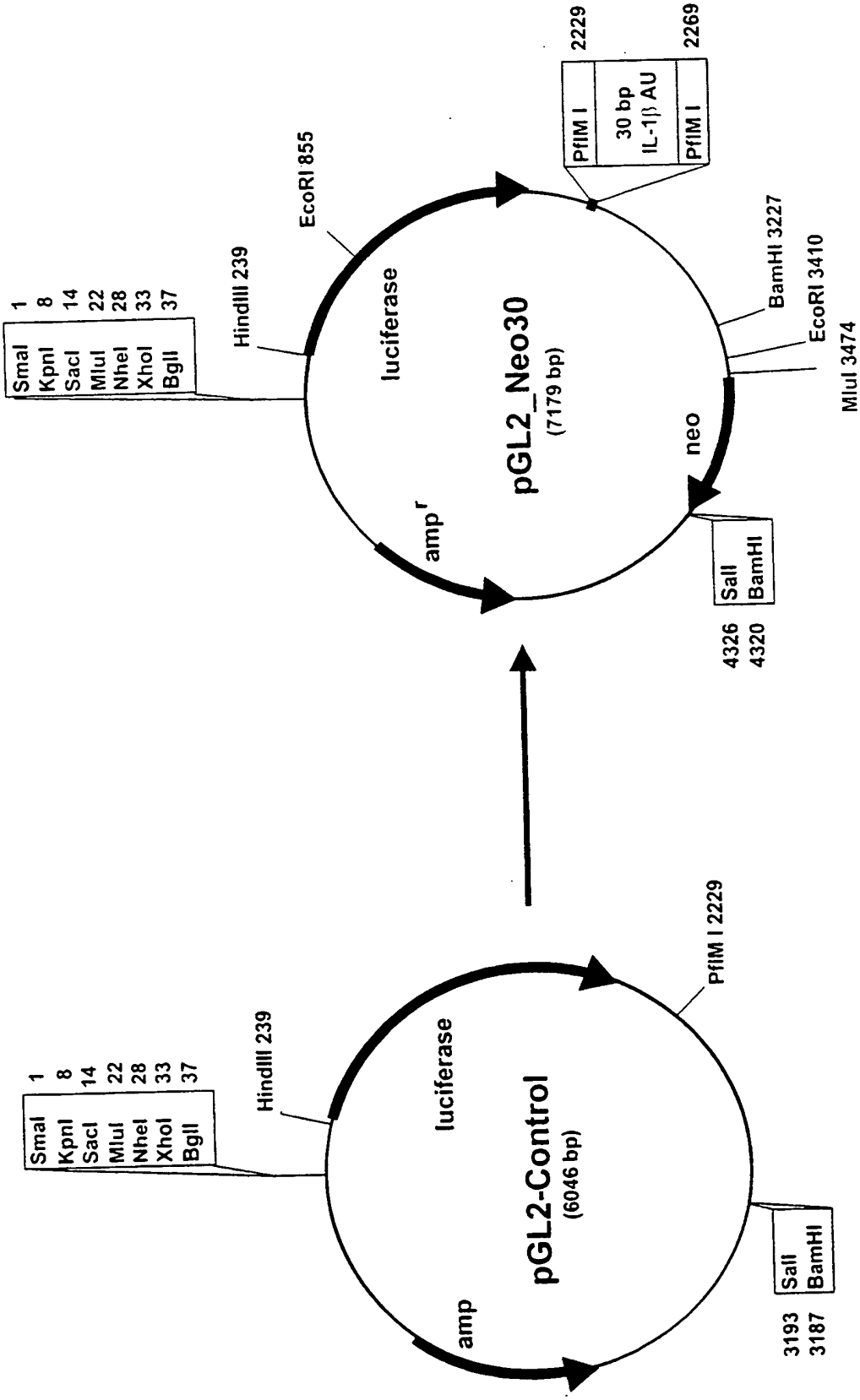

pGL2-Control
(6046 bp)
luciferase
amp
HindIII 239
PflM I 2229
SmaI 1
KpnI 8
SacI 14
MluI 22
NheI 28
XhoI 33
BglI 37
SalI 3193
BamHI 3187
pGL2_Neo30
(7179 bp)
luciferase
amp^r
neo
HindIII 239
EcoRI 855
PflM I 2229
30 bp IL-1β AU
PflM I 2269
BamHI 3227
EcoRI 3410
MluI 3474
SalI 4326
BamHI 4320

FIGURE 3

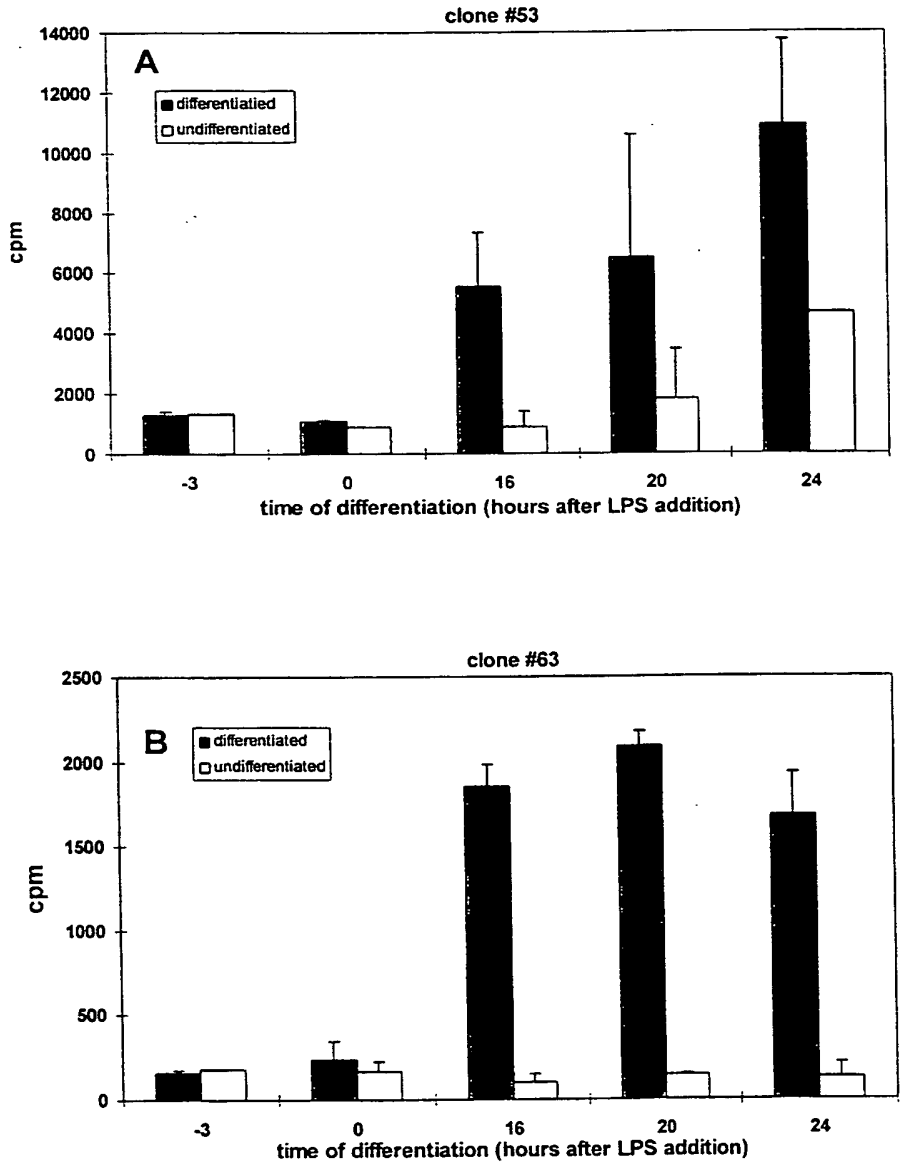

clone #53
A
differentiatied
undifferentiated
cpm
14000
12000
10000
8000
6000
4000
2000
0
-3
0
16
20
24
time of differentiation (hours after LPS addition)
clone #63
B
differentiated
undifferentiated
cpm
2500
2000
1500
1000
500
0
-3
0
16
20
24
time of differentiation (hours after LPS addition)

FIGURE 4

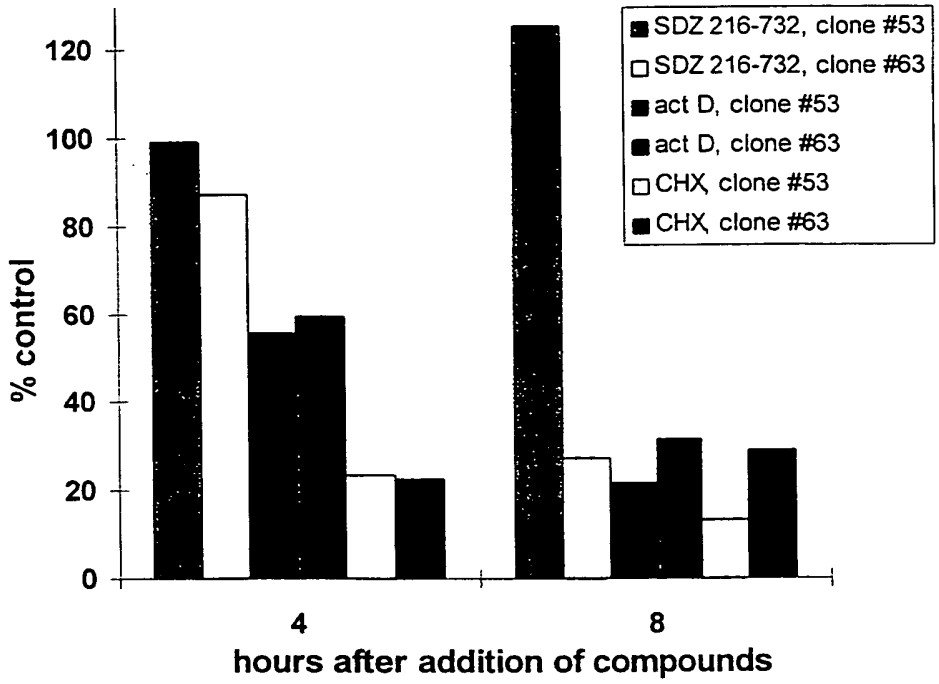

SDZ 216-732, clone #53
SDZ 216-732, clone #63
act D, clone #53
act D, clone #63
CHX, clone #53
CHX, clone #63
% control
120
100
80
60
40
20
0
4
8
hours after addition of compounds

FIGURE 5

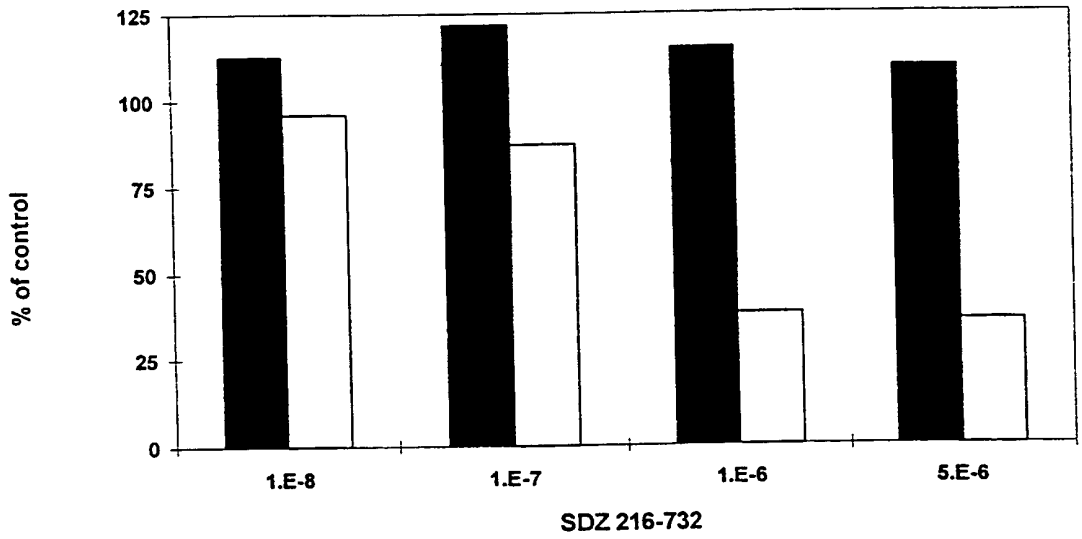


FIGURE 6

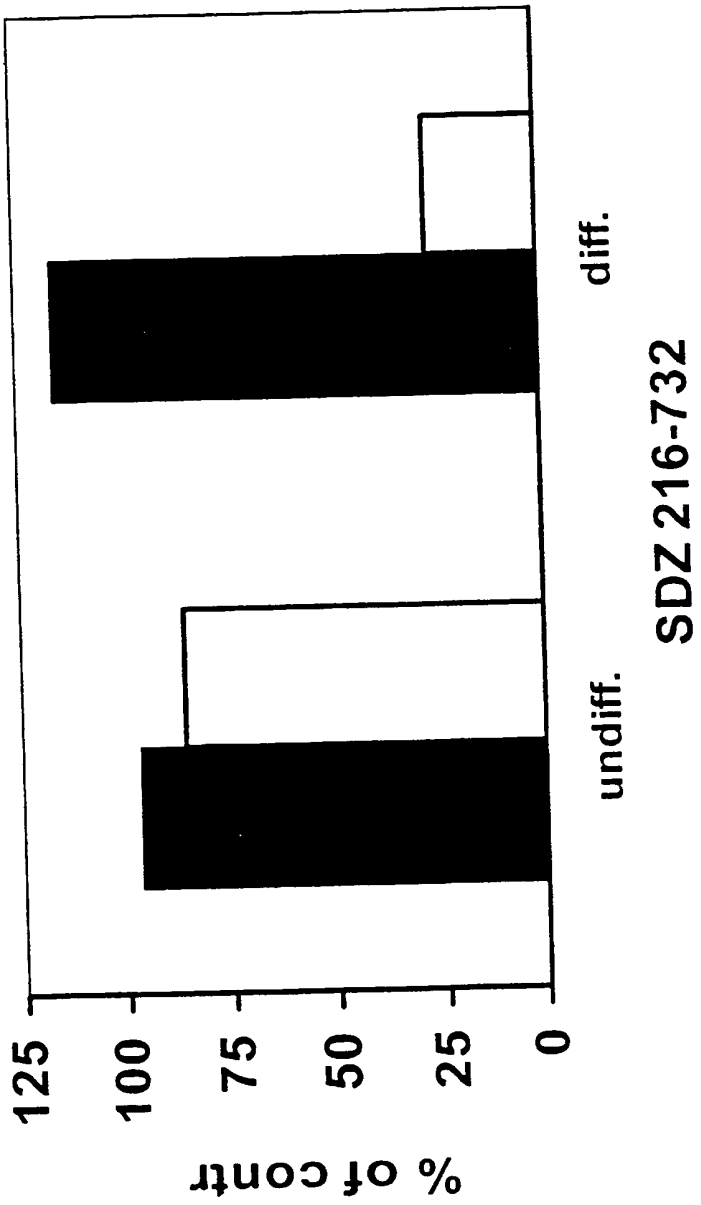


FIGURE 7

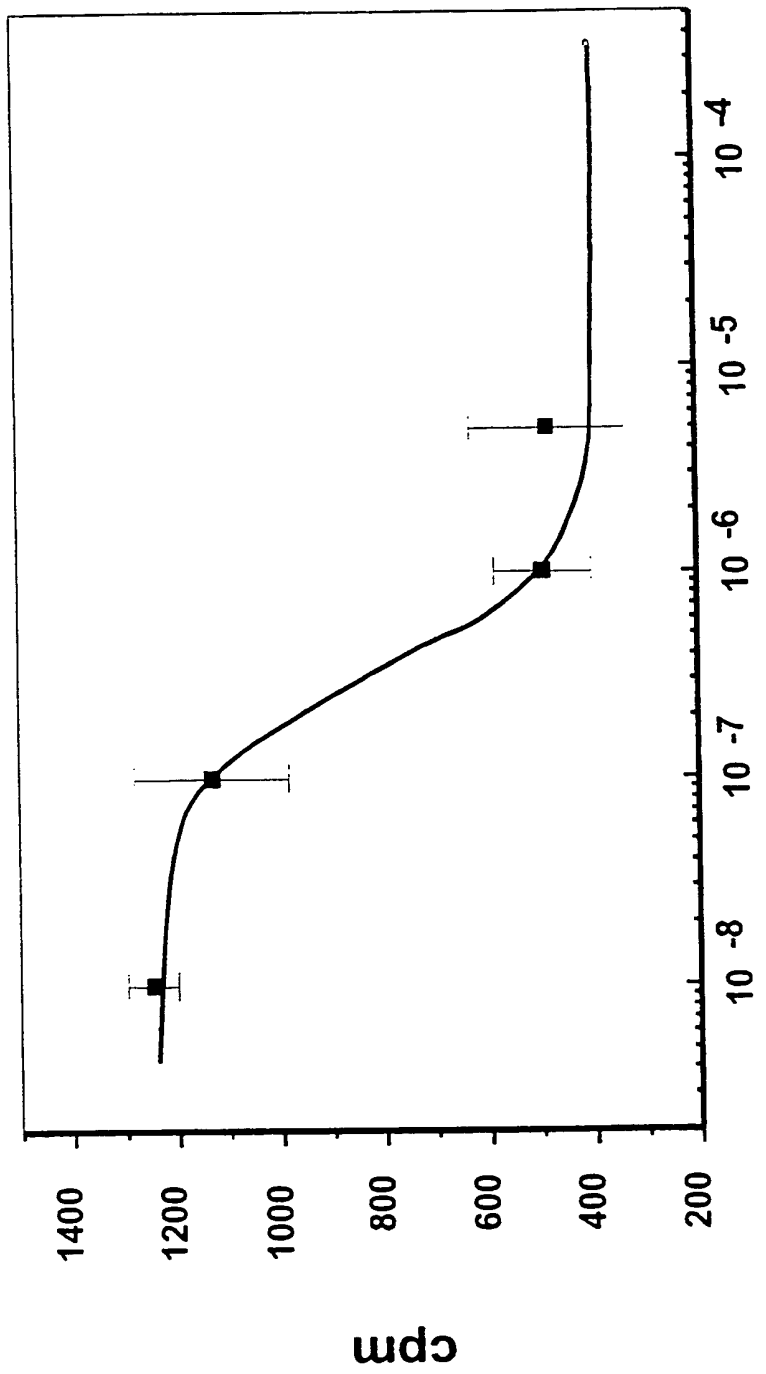

cpm
1400
1200
1000
800
600
400
200
10 -8
10 -7
10 -6
10 -5
10 -4
SDZ 216-732

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/01235

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 C12N5/10 C12Q1/68 A61K35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KASTELIC ET AL.: "INDUCTION OF RAPID IL-1BETA mRNA DEGRADATION IN THP-1 CELLS MEDIATED THROUGH THE AU-RICH REGION IN THE 3'UTR BY A RADICICOL ANALOGUE" CYTOKINE, vol. 8, no. 10, October 1996 (1996-10), pages 751-761, XP002138167 cited in the application the whole document	1-11
X	BANHOLZER ET AL.: "RAPAMYCIN DESTABILIZES INTERLEUKIN-3 mRNA IN AUTOCRINE TUMOR CELLS BY A MECHANISM REQUIRING AN INTACT 3'UNTRANSLATED REGION" MOLECULAR AND CELLULAR BIOLOGY, vol. 17, no. 6, 1997, pages 3254-3260, XP002138168 the whole document	1-11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 2000	06/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/01235

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 20212 A (US HEALTH ;PAVLAKIS GEORGE N (US); FELBER BARBARA K (US)) 14 October 1993 (1993-10-14) See page 25-page 30 the whole document	1-11
X	US 5 731 343 A (FENG LILI ET AL) 24 March 1998 (1998-03-24)	10,11
A	the whole document	1-9
A	WO 95 33831 A (CREATIVE BIOMOLECULES INC) 14 December 1995 (1995-12-14) the whole document	1-11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 99/01235

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9320212 A	14-10-1993	AU 678157 B	22-05-1997
		AU 3969493 A	08-11-1993
		CA 2132208 A	14-10-1993
		DE 69327456 D	03-02-2000
		EP 0635062 A	25-01-1995
		JP 7509121 T	12-10-1995
		US 5972596 A	26-10-1999
		US 5965726 A	12-10-1999
US 5731343 A	24-03-1998	AU 5175296 A	11-09-1996
		CA 2213632 A	29-08-1996
		EP 0810860 A	10-12-1997
		WO 9625928 A	29-08-1996
WO 9533831 A	14-12-1995	AU 703445 B	25-03-1999
		AU 2822395 A	04-01-1996
		CA 2191583 A	14-12-1995
		EP 0804573 A	05-11-1997
		JP 10505223 T	26-05-1998

PATENT COOPERATION TREATY

by fax and post

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ROBINSON, J.C.
Smart & Biggar
Box 11560 Vancouver Centre
650 W. Georgia St., Suite 2200
Vancouver, BC V6B 4N8
CANADA

FAX NO : (604) 682

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 29.03.2001
Applicant's or agent's file reference 82109-2	IMPORTANT NOTIFICATION
International application No. PCT/CA99/01235	International filing date *(day/month/year)* 23/12/1999
	Priority date *(day/month/year)* 24/12/1998
Applicant NOVATION PHARMACEUTICALS INC. et al.	

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel. +49 89 2399-8090

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
82109-2		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01235	23/12/1999	24/12/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/85		
Applicant		
NOVATION PHARMACEUTICALS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/07/2000	29.03.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Fotaki, M Telephone No. +49 89 2399 8709

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/01235

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-16 as originally filed

Claims, No.:

1-11 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

Sequence listing part of the description, pages:

1-3, filed with the demand

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3, 6-9
	No:	Claims	1, 2, 4, 5, 10, 11
Inventive step (IS)	Yes:	Claims	none
	No:	Claims	1-11
Industrial applicability (IA)	Yes:	Claims	1-10
	No:	Claims	11 reserved opinion

2. Citations and explanations
   see separate sheet

I. BASIS OF THE REPORT

1) Sequence listing pages 1-3 are also included in the basis of the report.

V. REASONED STATEMENT UNDER ARTICLE 35(2)

2) The following documents will be referred to:

D1: KASTELIC ET AL.: 'Induction of rapid IL-1beta mRNA degradation in thp-1 cells mediated through the AU-rich region in the 3'UTR by a radicicol analogue' Cytokine, vol. 8, no. 10, October 1996, pages 751-761;

D2: BANHOLZER ET AL.: 'Rapamycin destabilizes interleukin-3 mRNA in autocrine tumor cells by a mechanism requiring an intact 3' untranslated region' MOLECULAR AND CELLULAR BIOLOGY, vol. 17, no. 6, 1997, pages 3254-3260;

D3: WO 93 20212 A , 14 October 1993;

D4: US-A-5 731 343, 24 March 1998.

3) The subject-matter of the present application relates to the provision of assay systems whereby compounds may be identified which are capable of inducing instability of mRNA molecules. Candidate compounds are identified through their interaction with a nucleic acid sequence present in the 3' untranslated region of certain mRNA molecules, which sequence is known to confer instability to the bearing mRNA. Such instability sequences are AU rich elements (AREs) disclosed in documents D1, D2, D3 and references therein. Said documents disclose also expression systems comprising IL-1β or IL-3 gene sequences (D1 and D2, respectively) which comprise such AREs. Said expression systems are used in testing several compounds for their ability to induce degradation of mRNA.

4) The subject-matter of **Claims 1, 2, 4, 5** is not novel as required by Article 33(2) PCT.

Said claims relate to assay methods or systems which are disclosed in documents D1 and D2 as discussed above.

5) The subject-matter of **Claim 3** is not inventive as required by Article 33(3) PCT.

Said claim relates to a method of comparison of compounds inducing mRNA degradation (apparently for their ability to do so). Although such method is not disclosed in the above-mentioned documents its is directly derivable from their teachings and thus, not inventive.

6) The subject-matter of **Claims 6-9** is not inventive as required by Article 33(3) PCT.

Said claims disclose an assay system for the identification of compounds which induce mRNA degradation which comprises an expression system with two components, one comprising a gene comprising the instability inducing region and another which is lacking said region. Document D3 discloses expression systems lacking said instability inducing region. Combination of the teachings of document D3 with the teachings of document D1 or D2 will result in the claimed subject-matter. The skilled person would have incentive to combine the two documents since they refer specifically to compounds that induce mRNA degradation.

7) The subject-matter of **Claim 10** is not novel as required by Article 33(2) PCT.

The claimed subject-matter is drafted as a product by process. However, under the definition fall a number of compounds which are already known (for example the compounds disclosed in documents D1 and D2). A known compound is not rendered novel merely by the fact that it is produced by means of a specific process even if said process is novel.

8) The subject-matter of **Claims 11** is not novel as required by Article 33(2) PCT.

The claimed use is disclosed in document D4.

9) For the assessment of the present **Claim 11** as far as it is directed to a method of treatment of the human or animal body or to a diagnostic method practised on the human or animal body, no unified criteria exist in the PCT, on the question whether they are industrially applicable. The patentability can be dependent upon the formulation of the claim.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ROBINSON, J. Christopher
SMART & BIGGAR
Box 11560, Suite 2200
650 West Georgia Street
Vancouver, BC V6B 4N8
CANADA

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	02.04.2001
Applicant's or agent's file reference 82109-1	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01234	23/12/1999	24/12/1998

Applicant
NOVATION PHARMACEUTICALS INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Exner, K Tel.+49 89 2399-7826

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
82109-1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01234	23/12/1999	24/12/1998
International Patent Classification (IPC) or national classification and IPC A61K31/365		
Applicant NOVATION PHARMACEUTICALS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/07/2000	02.04.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Stoltner, A Telephone No. +49 89 2399 8408

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/01234

I. Basis of the report

1. With regard to the elements of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-27 as originally filed

Claims, No.:

1-10 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 2,4,7,9.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 2,4,7,9 (method of treatment).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims

	No:	Claims 1-10
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-10
Industrial applicability (IA)	Yes:	Claims 1-10
	No:	Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

ad section III:

1). Claims 2, 4, 7 and 9 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

ad section V:

1). The present application concerns the use of a compound inducing degradation of mRNA for use as a pharmaceutical, explicitly excluding the compound "radicicol A".

2). Following documents, considered as relevant prior art, are discussed with context to the subject-matter of the present application:

D1, WO-A-9 625 928, relates to radicicol or functional derivatives therefrom for treating immunopathological disorders associated with the expression of cytokines like IL-1 and TNF-alpha (cf. abstract, page 5, lines 18-27, page 10, last para. bridging with page 11, lines 1-13). D1 apparently recognises the fact, that the expression of the genes encoding cytokines (=proinflammatory agents) leads to the outset of immunopathological disorders (cf. page 14 2nd para. and page 17, lines 5-26). This is further documented on page 20, 2nd full paragraph where the stability of the mRNA is measured by increase/decrease of the kinase activity. The teaching of D1 thus clearly reveals that radicicol and its derivatives (other than radicicol A) use the mechanism of mRNA degradation for the treatment of diseases related to mRNA in the conditions as presently described. This unambiguously can be derived from the referred diseases which are identical to those listed in the present application.

D2, Advances in Experimental Medicine and Biology, 407, 1996, pp. 281-288, L. Feng et al., reports that the protein tyrosine kinase inhibitor radicicol post-transkriptionally inhibits the expression of COX-2 and of pro-inflammatory cytokines by accelerating the degradation of mRNA (cf. introduction and page

283, 2nd and 4th paras., and in particular page 384, 2nd para. bridging with page 385, first para.). However, the pharmaceutical use of radicicol is not suggested in D2.

D3, EP-A-606 044, cited in the application, discloses radicicol derivatives as cytokine release inhibitors and their medical use in treating inflammatory diseases such as septic shock, psoriasis and asthma (cf. abstract, page 6, lines 49-51, page 8, lines 29-32).

D4, US-A-5 597 846, discloses radicicol derivatives with antitumoral activity (cf. abstract, col. 1, lines 30-39.

D5, EP-A-460 950, in a similar way, discloses antitumoral activity of radicicol derivatives (cf. abstract and page 2, lines 12-30.
D4 and D5, however, are silent on mechanism (degradation of mRNA) by which radicicol exerts its activity in the treatment of diseases associated with excessive cytokine release.

3). In view of the cited documents, the subject-matter of the present application appears to be anticipated by the prior art. It is known that radicicol (and its derivatives including the analogue "A") also degrade the mRNA necessary for the expression of cytokines which by themselves are the origin of various inflammatory diseases as presently depicted. As is more, the use of radicicol and its derivatives other than analogue "A" in the therapy of diseases such as psoriasis, septic shock or asthma is expressly mentioned in D1. As consequence, the subject-matter of the present application clearly fails to comply with the requirements of novelty and inventive step pursuant to Arts. 33(2) and 33(3) PCT.

4). For the assessment of the present claims 2, 4, 7 and appending claim 8 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of

a medicament for a new medical treatment. The same applies to the corresponding passages throughout the application.

ad section VIII:

1). The subject-matter of claim refers to nonsense.